CHAPTER 4

RESULTS

4.1 Design and evaluation of subtype-specific primers

The MSSP assay was developed to be an effective and useful tool to monitor HIV-1 strains in Southeast and South Asia including China. The assay used 7 pairs of universal outer primers and 24 pairs of subtype-specific inner primers distributed along 8 different regions of the HIV-1 genome to capture subtypes B, C, CRF01_AE and their recombinant forms (CRFs and URFs). Eighty-eight primers in total were designed and synthesized. PCR reactions were performed in each region using relevant designed primers. First round primers were selected considering the positive bands given for all three subtypes. The second round primers that gave strongly positive band with the positive subtype, and did not show cross-reactive with the other two subtypes were selected for the developed assay. There were 62 primers, 14 were for first round amplification and 48 primers were chosen to differentiate subtype B, C, CRF01_AE and their recombinant forms. The primers were designed to sit between breakpoints of the recombinant genomes with the goal that the MSSP assay would be effective enough to detect all 16 recently identified recombinants in Thailand and neighboring countries. The sequences and details of primers used in the MSSP assay are shown in Table 4.1.

Table 4.1 Primers used in the MSSP Assay for CRF01_AE, subtype B, and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/reverse	Tm (^{0}C)	Size (bp)
Gag						
Outer primers						
	GKTH04F02	995 – 1022	AGACAGGAWCAGARGAACTTARATCATT	Forward	62.0	262
	GKTH04R03	1256 – 1232	ACCCATGCATTYAAAGTTCTAGGTG	Reverse	64.8	
Inner primers						
CRF01_AE	SS04FE05	1154 – 1170	CAGGAAGCAGCAGCATA	Forward	58.7	63
	SS04RE06	1216 –1201	CCATTTGCCCTTGTGC	Reverse	61.3	
Subtype B	SS04FB05	1152 – 1170	CACAGGAAACARCAGCCCG	Forward	66.7	60
	SS04RB06	1211 –1197	TGCCCCTGGAGGATT	Reverse	60.1	
Subtype C	SS04FC03	1141 – 1158	AAAGAGGCTGACGGGAAG	Forward	61.9	75
	SS04RC06	1215 – 1197	CATTTGCCCTTGGAGACTC	Reverse	62.2	

[a] Deliberately induced mismatches are red color

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B, and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/ reverse	Tm (^{0}C)	Size (bp)
Pol Protease						
Outer primers						
	SS01FU01	1727-1751	TAAAAAAAYTGGATGACAGAMACCTT	Forward	60.5	665
	GKTH01R05	2391-2367	TCATTTTTGGTTTCCATYTTCCTGG	Reverse	68.9	
Inner primers						
CRF01_AE	SS01FE04	1885 – 1903	CAATGAGCCAMGCACTAC	Forward	57.6	428
	SS01RE02	2312 – 2295	TTTYAGCTGTCCTCCTAATT	Reverse	54.7	
Subtype B	SS01FB04	1930 – 1953	GGCAATTTTAGGAAYCAAAGACAG	Forward	63.5	384
	SS01RB04	2313 – 2294	CCTTTAATTGCCCCCCTTTC	Reverse	65.2	
Subtype C	SS01FC04	1925 – 1945	AGAGAAGCAATTTTAAAGGCTCT	Forward	60.4	386
	SS01RC02	2310 – 2293	TTATCTGGCCCCCTAATC	Reverse	58.8	

[a] Deliberately induced mismatches are red color.

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B , and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/reverse	Tm (°C)	Size (bp)
Pol RT						
Outer primers						
	VWTH05UF01	2808 – 2833	TTYTGGGAAGTTCAATTAGGAATACC	Forward	64.6	518
	SS05RU01	3325 – 3303	TTCTGTATRTCATTGACAGTCCA	Reverse	60.9	
Inner primers						
CRF01_AE	SS05FE03	3042 – 3069	ACAARAATCTTAGAGCCCTTTAGAAKAA	Forward	60.5	146
	SS05RE03	3187 – 3168	CCCCAGGTCAATAGATGAGC	Reverse	63.3	
Subtype B	SS05FB03	3042 – 3067	ACAAAAATCTTAGAGCCTTTTAGTAA	Forward	58.7	143
	SS05RB03	3184 - 3161	CACYTCAACAGATGTTGTCTCCGT	Reverse	66.3	
Subtype C	SS05FC03	3048 – 3067	ATCTTAGAGCACTTTAGGGC	Forward	56.7	138
	SS05RC03	3185 – 3162	CCCACYTTAACAGATGTTBTCTAAA	Reverse	61.1	

[a]Deliberately induced mismatches are red color.

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B, and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/ reverse	Tm (^{0}C)	Size (bp)
Pol Integrase						
Outer primers						
	Pol J	4371 –4392	GAAGCCATGCATGGACAAGTAG	Forward	65.6	382
	GKTH06R03	4752 –4731	CTGTCTTAAGRTGYTCAGCTTG	Reverse	58.3	
Inner primers						
CRF01_AE	SS06FE01	4511 –4532	ACAGGAGACAGCATACTTTTTG	Forward	59.8	137
	SS06RE01	4647 –4628	CAAATTCCTGTYGGACATTG	Reverse	60.4	
Subtype B	SS06FB01	4511 –4532	GCAGGAAACAGCATACTTTGTC	Forward	62.5	134
	SS06RB01	4644 –4628	ATTCCTGCTTGATCGCC	Reverse	61.4	
Subtype C	SS06FC01	4507 –4532	CAGGACAAGAAACAGCATACTTTATA	Forward	61.2	146
	SS06RC01	4652 –4528	AATTCGAAATTCCTGTTGGATAACT	Reverse	63.5	

[a] Deliberately induced mismatches are red color.

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B, and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/reverse	Tm (^{0}C)	Size (bp)
Vpr						
Outer primers						
	VWTH02UF01	5749 –5774	TGCAACAACTRCTGTTTRTTCATTTC	Forward	65.8	391
	VWTH02UR01	6139 –6111	TACTATRGTCCACACACAACTATTGCTASGA	Reverse	66.7	
Inner primers						
CRF01_AE	SS02FE04	5795 –5816	GCAGAATAGGCATTATACCAGG	Forward	61.2	147
	SS02RE03	5941 –5925	AGCATARTTGGCAATACC	Reverse	54.9	
Subtype B	SS02FB05	5790 –5813	ACATAGCAGAATAGGCATTAGTCA	Forward	60.5	155
	SS02RB03	5944 –5925	GAAACAAACTTGGCAATCAA	Reverse	60.1	
Subtype C	SS02FC05	5795 –5813	GCAGAATAGGCATTTAGCC	Forward	58.9	152
	SS02RC03	5946 –5925	TGAAAGCAAACTAGACAATAGT	Reverse	54.9	

[a] Deliberately induced mismatches are red color.

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B , and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/reverse	Tm (°C)	Size (bp)
Gp 120						
Outer primers						
	GKTH07F03	6955 – 6981	CAGTACAATGYACACATGGAATTARGC	Forward	63.9	847
	SS08RU01	7801 – 7783	GCTGCTCCYAAGAACCCAA	Reverse	61.9	
Inner primers						
CRF01_AE	SS09FE01	7151 – 7180	HMTATAGGACCAGGACAAGTATTCTACAG	Forward	63.3	381
	SS08RE05	7531 – 7510	GCATACATTGCTTGTCCTRTTC	Reverse	62.3	
Subtype B	SS09FB03	7021 – 7049	CAGAAGAAGAGGTAGTAATTATATCTAGC	Forward	58.4	304
	SS08RB02	7324 – 7302	CCTCCTGAGGATTGMTTAAACAC	Reverse	62.5	
Subtype C	SS09FC02	7083 – 7109	CATCTTAATCAATCTGTAGAAATTGTG	Forward	60.6	231
	SS08RC01	7313 – 7283	TGRWKCAAATTTTATTGTTTTATTAGGG	Reverse	61.8	

[a] Deliberately induced mismatches are red color.

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B , and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/reverse	Tm (°C)	Size (bp)
Rev						
Outer primers						
	GKTH03F02	8520 – 8544	TTCAGCTACCACCGCTTGAGAGACT	Forward	70.0	657
	VWTH10R03	9176 – 9155	CARTCAGGGAAGWAGCCTTGTG	Reverse	64.6	
Inner primers						
CRF01_AE	SS03FE02	8586 – 8607	AGGAGTCTGAAGGGACTGAGAC	Forward	62.7	133
	SS03RE02	8718 – 8695	CTTCTATAACCCTATCTGTCCACC	Reverse	60.6	
Subtype B	SS03FB03	8576 – 8589	TCTGGGACGCACGG	Forward	61.8	120
	SS03RB01	8695 – 8669	TCAGGTACTGCTATAGCTGTGGVAATG	Reverse	65.0	
Subtype C	SS03FC01	8545 – 8567	TCATATTAGTGACAGCGAGAGTG	Forward	60.9	222
	SS03RC03	8766 – 8744	TTATTCTTCTAGGTATGTTGCGG	Reverse	60.8	

[a] Deliberately induced mismatches are red color.

Table 4.1 (continued) Primers used in the MSSP Assay for CRF01_AE, subtype B , and subtype C

Region	Name	HXB2 numbering	Sequence (5'----------> 3')[a]	Forward/ reverse	Tm (°C)	Size (bp)
Nef						
Outer primers						
	GKTH03F02	8520 – 8544	TTCAGCTACCACCGCTTGAGAGACT	Forward	70.0	657
	VWTH10R03	9176 – 9155	CARTCAGGGAAGWAGCCTTGTG	Reverse	64.6	
Inner primers						
CRF01_AE	SS10FE01	8847 – 8868	GGAAAGAATAARGCAAACTCCT	Forward	60.0	114
	SS10RE01	8960 – 8939	CAGACACAATCAGCATTATTCA	Reverse	60.2	
Subtype B	SS10FB01	8840 – 8863	GKGTAAGGGAAAGAATGRAACTAG	Forward	56.7	123
	SS10RB01	8962 – 8942	CACAAGCGGCATTAGTAGCTG	Reverse	64.6	
Subtype C	SS03FC05	8725 – 8746	CAARGAAYTTGTAGAGCTATCAG	Forward	57.2	238
	SS10RC01	8962 – 8942	CACAATCAGCATTAGTGGTGT	Reverse	60.1	

[a]Deliberately induced mismatches are red color.

4.2 The initial evaluation of the assay (sensitivity and subtype specificity)

For the initial evaluation of the assay sensitivity and subtype specificity, near full-length genome PCR products of CRF01_AE (02TH.OUR737I and 99TH.OUR199I), co-cultured PBMC DNA of subtype B (96TH_NP1538 and NP1635) and near full-length genome cloned of subtype C (95IN21068 and 93IN905) were used as DNA template. The length of the PCR products in first round PCR were 262-847 base pairs (bp) and in the second round were 60-428 bp. The given positive PCR band from each subtype was shown in Figure 4.1. Figures 4.2 to 4.7 showed the performance of MSSP assay to differentiate three HIV-1 subtypes in 8 genome regions.

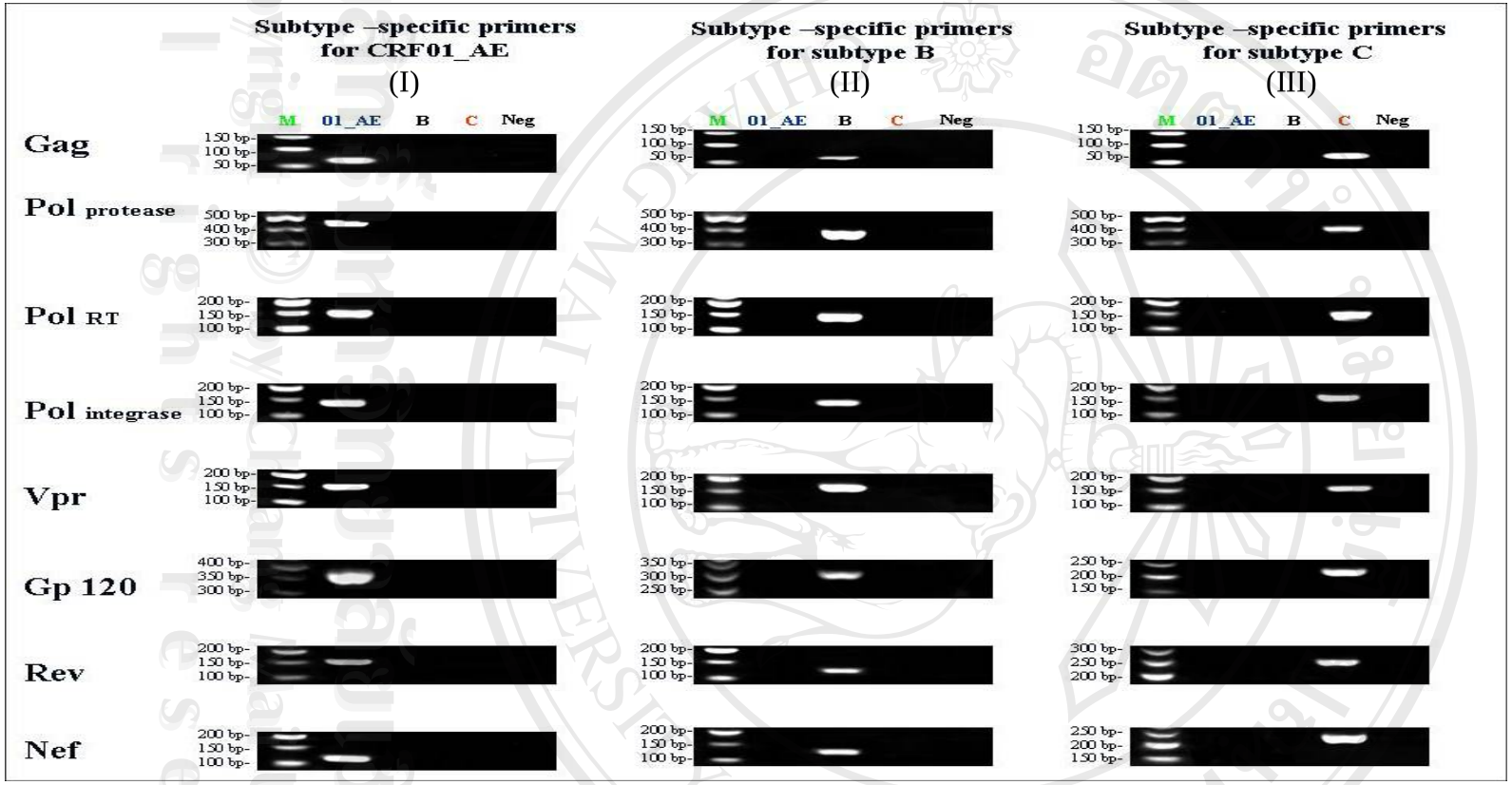

Figure 4.1 The results of initial evaluation of the MSSP assay in each region of HIV genome. (I) Gel profile of amplified product by subtype-specific primer for CRF01_AE. (II) Gel profile of amplified product by subtype-specific primer for subtype B. (III) Gel profile of amplified product by subtype-specific primer for subtype C. Lane M : DNA marker, Lane 01_AE: CRF01_AE strain, Lane B: Subtype B strain, Lane C: Subtype C strain, Lane Neg: Negative control

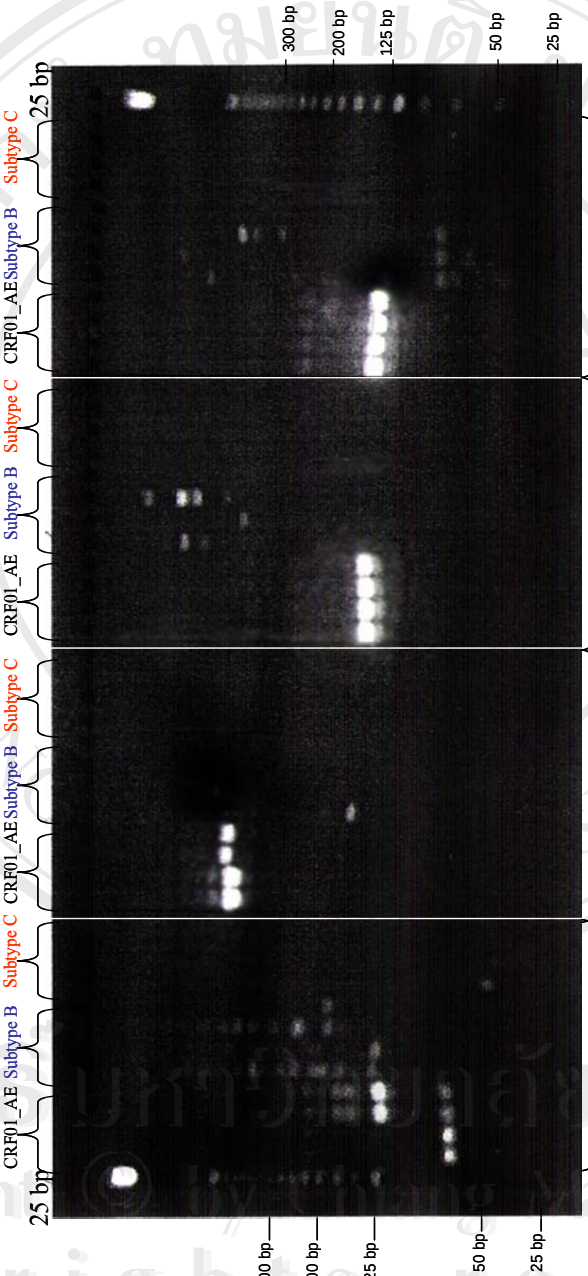

Figure 4.2 MSSP assay evaluation with reference strains using subtype specific CRF01_AE primers in *Gag*, *Pol PR*, *Pol RT*, and *Pol IN* region

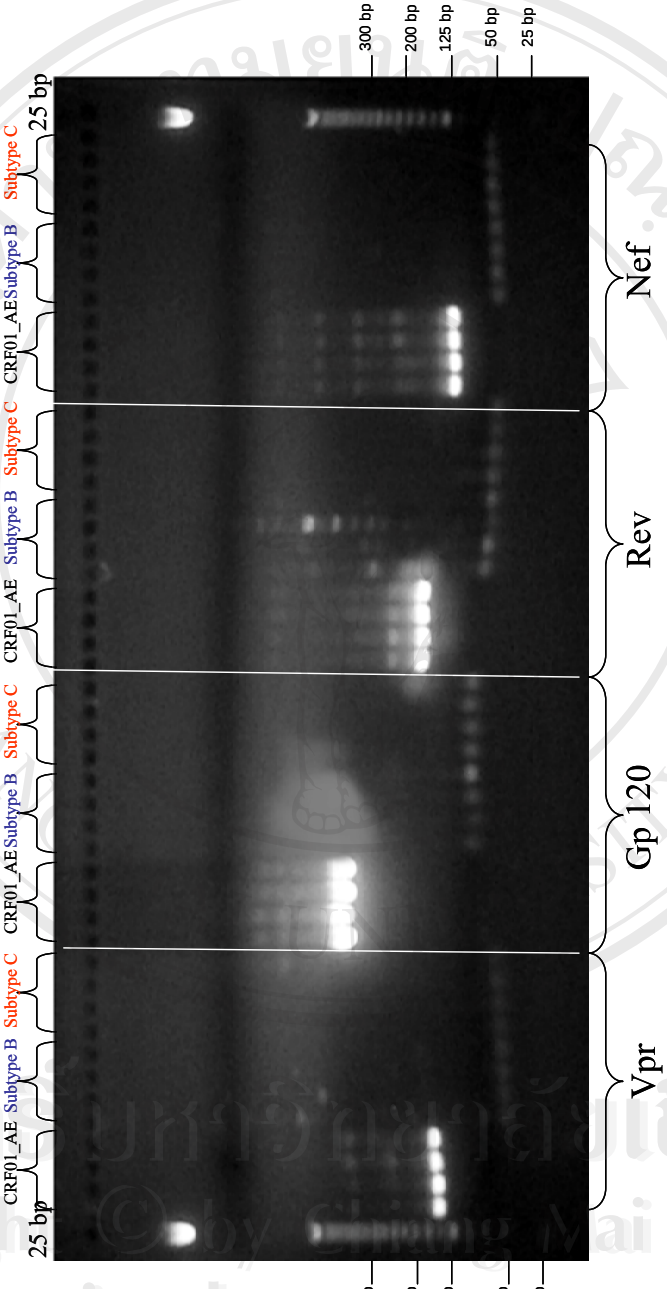

Figure 4.3 MSSP assay evaluation with reference strains using subtype specific CRF01_AE primers in *Vpr*, *Gp120*, *Rev*, and *Nef* region

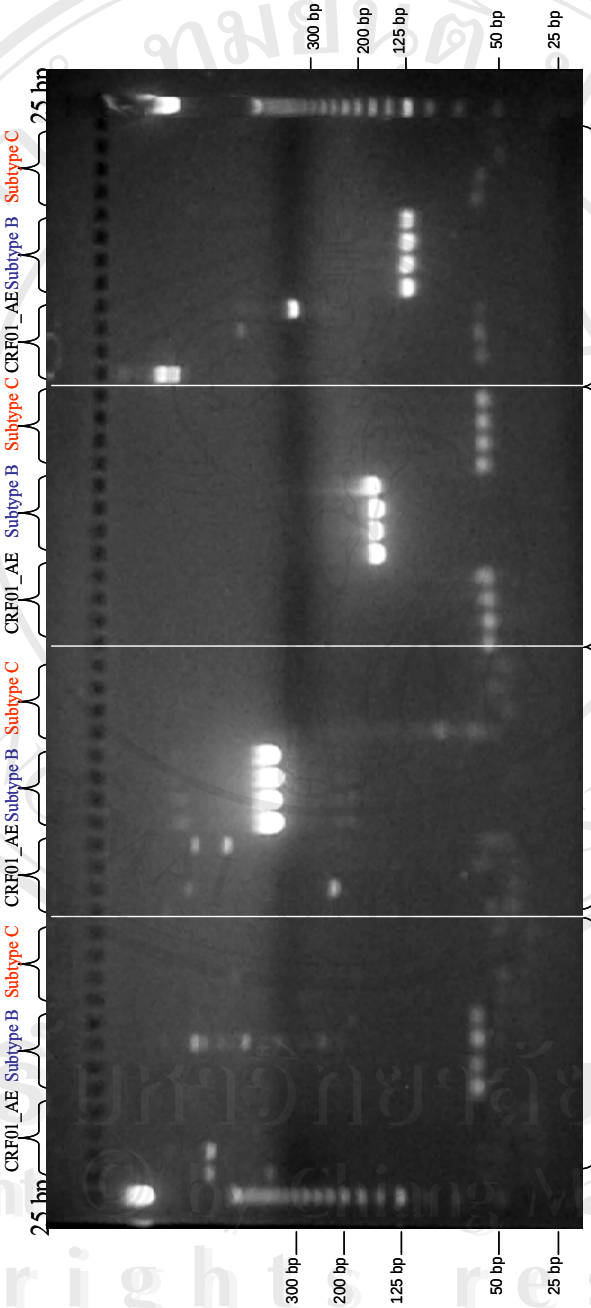

Figure 4.4 MSSP assay evaluation with reference strains using subtype specific B primers in *Gag, Pol PR, Pol RT,* and *Pol IN* region

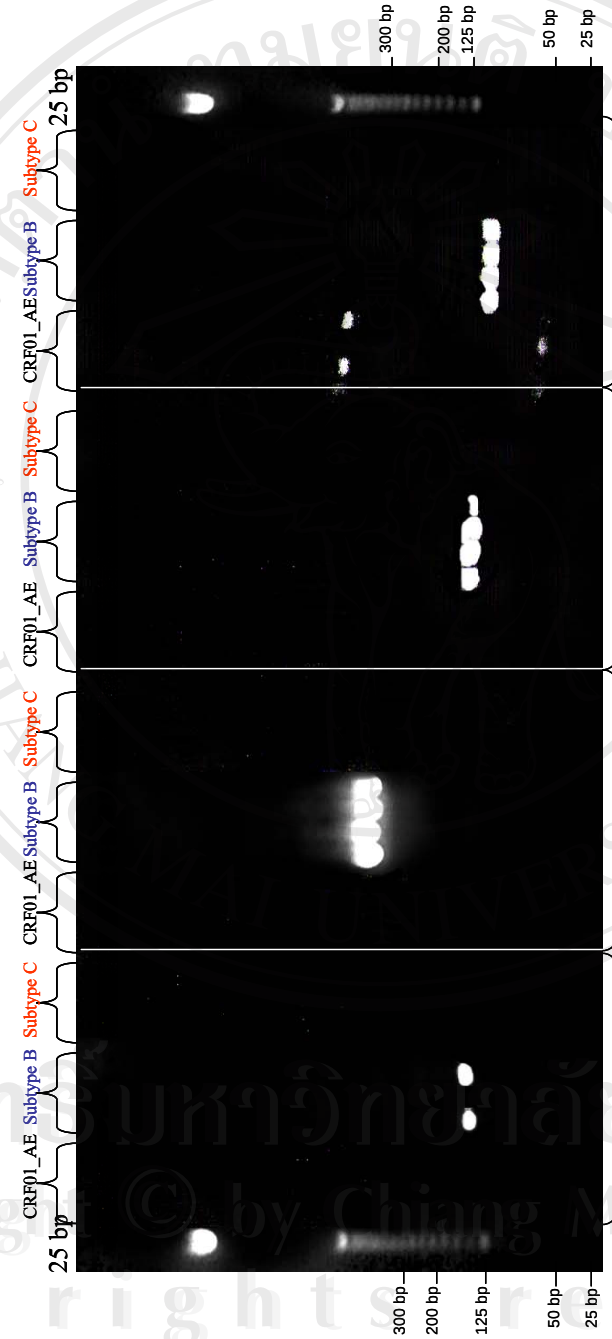

Figure 4.5 MSSP assay validation with reference strains using subtype specific B primers in *Vpr*, *Gp120*, *Rev*, and *Nef* region

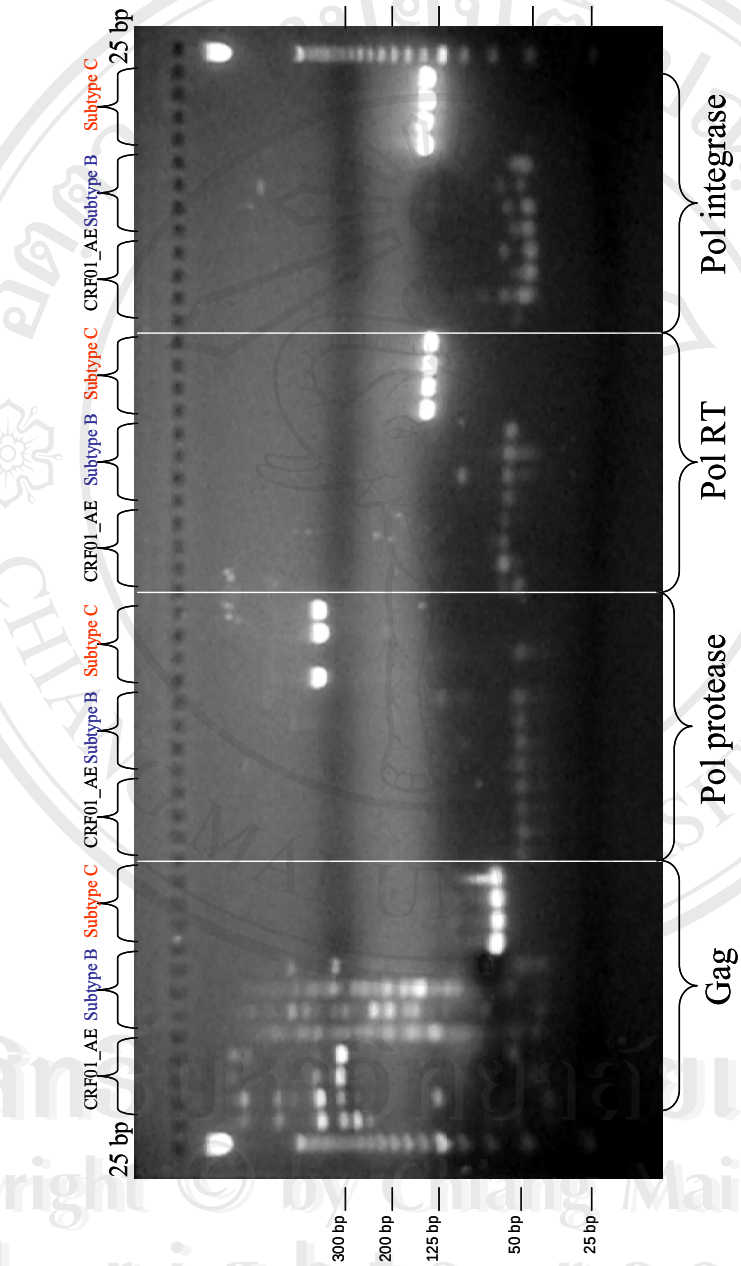

Figure 4.6 MSSP assay evaluation with reference strains using subtype specific C primers in *Gag, Pol PR, Pol RT*, and *Pol IN* region

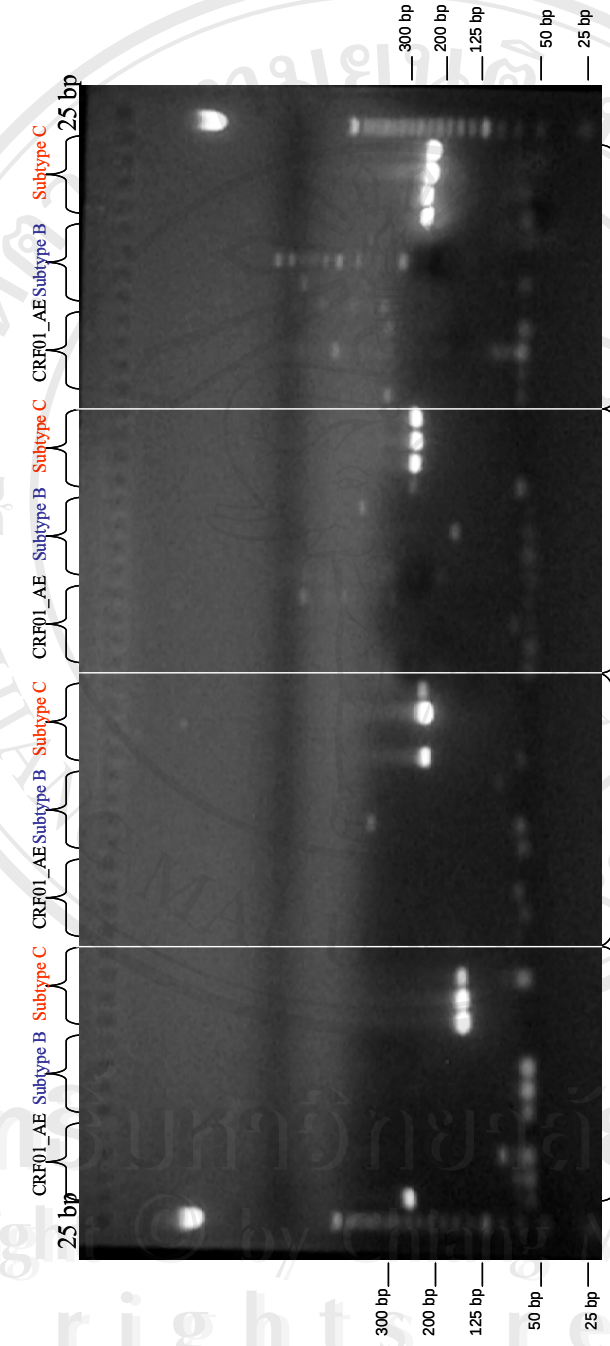

Figure 4.7 MSSP assay validation with reference strains using subtype specific C primers in *Vpr, Gp120, Rev,* and *Nef* region

4.3 MSSP assay evaluation on clinical samples

The MSSP assay was performed on the panel of 41 DNA samples. The represented HIV-1 subtypes were CRF01_AE (n=33), CRF15_01B (n=2) and unique CRF01_AE/B recombinants (n=6). The virtually full-length genome sequences of these HIV-1 strains were previously obtained. The MSSP results of this HIV-1 DNA panel were shown in Table 4.2. The structure of the HIV-1 genome and performances of MSSP assay on their genome regions were shown in Figure 4.8.

The MSSP assay was performed on these DNA samples totaled 328 genome regions. From these results, the sensitivity and specificity of the assay were calculated from the 328 genome regions. Figure 4.9 showed sensitivity of the MSSP assay on the panel of 41 clinical DNA samples in each genome region. Table 4.3 showed the sensitivity and specificity of primers in MSSP assay. Since the cross reactive in each region of these samples were rarely seen between subtypes, and non-specific amplification was not seen in the negative samples, the MSSP assay provided 100% specificity overall. The sensitivity of MSSP assay in each region was in a range of 73-100% which were 90, 73, 93, 95, 76, 100, 95, and 98 % for *Gag (p17-p24), Pol (p2-p7 p6 protease), Pol (p51 RT), Pol (p31 integrase), Vpr, Env (gp120), Rev* and *Nef*, respectively.

Table 4.2 The results of MSSP assay on a panel of 41 clinical DNA samples

			Gag	Pol PR	Pol RT	Pol IN	Vpr	Env	Rev	Nef
1	99TH.OUR008 I	CRF01_AE				E		E	E	E
2	99TH.OUR044 I	CRF01_AE	E	E		E	E	E	E	E
3	99TH.OUR066 I	CRF01_AE		E	E	E		E	E	E
4	99TH.OUR164 I	CRF01_AE	E	E	E	E	E	E	E	E
5	99TH.OUR098 I	CRF01_AE	E	E	E	E	E	E	E	E
6	99TH.OUR422 I	CRF01_AE	E	E	E	E	E	E	E	E
7	99TH.OUR202 I	CRF01_AE	E	E	E	E		E	E	E
8	99TH.OUR203 I	CRF01_AE	E		E	E	E	E	E	E
9	99TH.OUR258 I	CRF01_AE	E	E	E	E	E	E	E	E
10	99TH.OUR199 I	CRF01_AE	E		E	E	E	E	E	E
11	00TH.OUR057 I	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12	00TH.OUR595 I	CRF01_AE	E	E	E	E	E	E	E	E
13	00TH.OUR661 I	CRF01_AE	E	E	E	E	E	E	E	E
14	00TH.OUR200 I	CRF01_AE	E		E	E	E	E	E	E
15	00TH.OUR201 I	CRF01_AE	E		E	E		E	E	E
16	00TH.OUR721 I	CRF01_AE	E	E	E	E	E	E	E	E
17	00TH.OUR724 I	CRF01_AE	E		E		E	E	E	E
18	00TH.OUR736 I	CRF01_AE	E	E	E	E	E	E	E	E
19	00TH.OUR746 I	CRF01_AE	E	E	E	E	E	E	E	E
20	00TH.OUR810 I	CRF01_AE	E	E		E	E	E	E	E
21	01TH.OUR642 I	CRF01_AE	E		E	E	E	E	E	E

n.a = Not available sample

Table 4.2 (Continued) The results of MSSP assay on a panel of 41 clinical DNA samples

			Gag	Pol PR	Pol RT	Pol IN	Vpr	Env	Rev	Nef
22	01TH.OUR647 I	CRF01_AE	E		E	E	E	E	E	E
23	01TH.OUR674 I	CRF01_AE	E	E	E			E		
24	01TH.OUR700 I	CRF01_AE	E	E	E	E	E	E	E	E
25	01TH.OUR033 I	CRF01_AE/B	E	B	E	E	B	E	B	E
26	01TH.OUR598 I	CRF01_AE		E	E	E	E	E	E	E
27	01TH.OUR609 I	CRF01_AE	E	E	E	E		E	E	E
28	01TH.OUR786 I	CRF01_AE	E	E	E	E		E		E
29	01TH.OUR414 I	CRF01_AE	E		E	E	E	E	E	E
30	01TH.OUR788 I	CRF01_AE	E	E	E	E	E	E	E	E
31	01TH.OUR702 I	CRF01_AE	E	E	E	E	E	E	E	E
32	01TH.OUR830 I	CRF01_AE	E	E	E	E	E	E	E	E
33	02TH.OUR658 I	CRF01_AE	E	E	E	E	E	E	E	E
34	02TH.OUR846 I	CRF01_AE/B		B	E	E	B	E	B	E
35	02TH.OUR737 I	CRF01_AE	E	E	E	E		E	E	E
36	02TH.OUR740 I	CRF01_AE/B	B	B	B	E		E	B	B
37	02TH.OUR769 I	CRF01_AE	E	E	E	E	E	E	E	E
38	02TH.OUR847 I	CRF01_AE/B	E		E	E	E	E	B	E
39	02TH.OUR840 I	CRF01_AE/B	E	E	E	E	E	E	E	E
40	02TH.OUR 1331	CRF15_01B	E	E	E	E	E	B	E	E
41	02TH.OUR 1332	CRF15_01B	E		E	E	E	B	E	E
42	02TH.OUR 2574	CRF01_AE/B	B	B	E	B		E	B	B

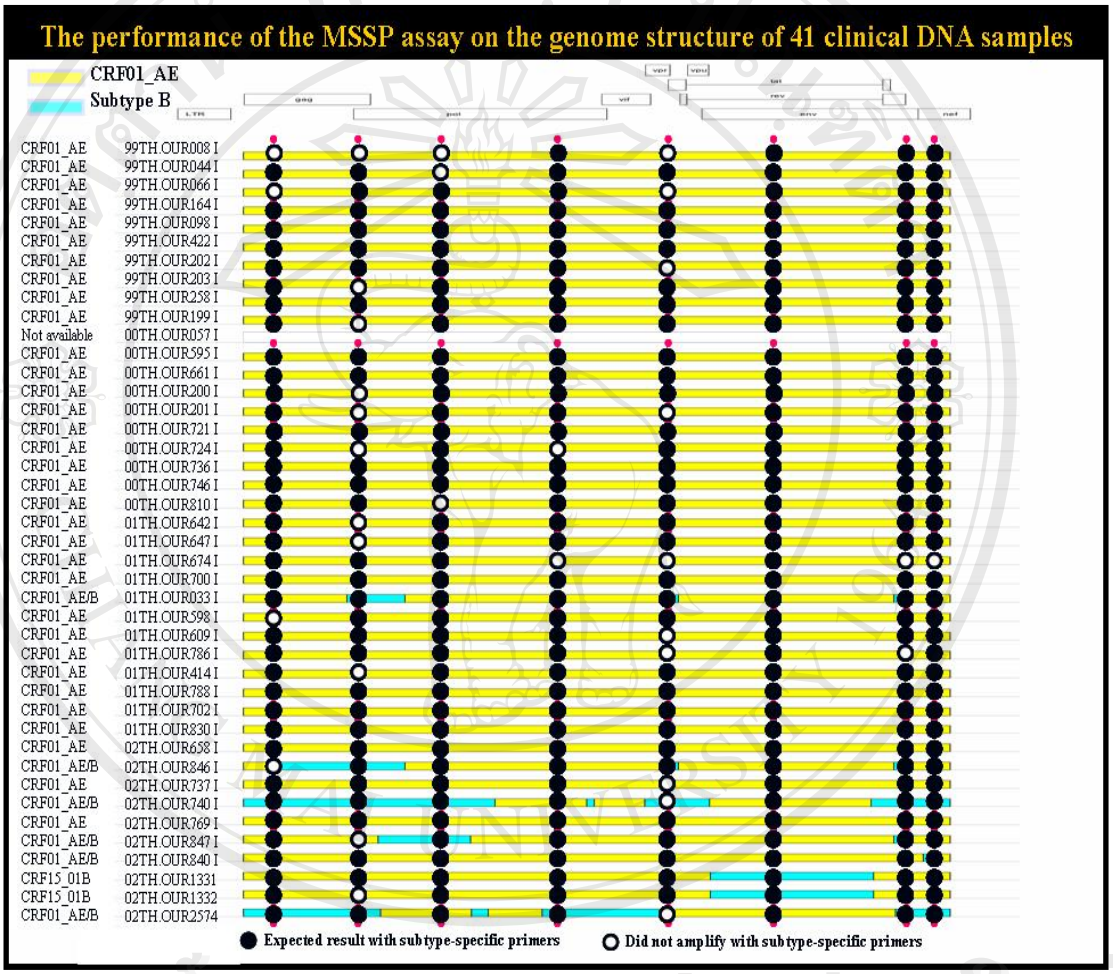

Figure 4.8 The performance of the MSSP assay on the genome structure of 41 clinical DNA samples. HIV-1 strains and the subtype characterized by full-genome sequencing are presented. The genome structures of HIV-1 strains are shown with the location of breakpoints according to the diagram of HIV-1 genome at the top. Genome segment in yellow indicates CRF01_AE and in blue indicates subtype B genetic material.

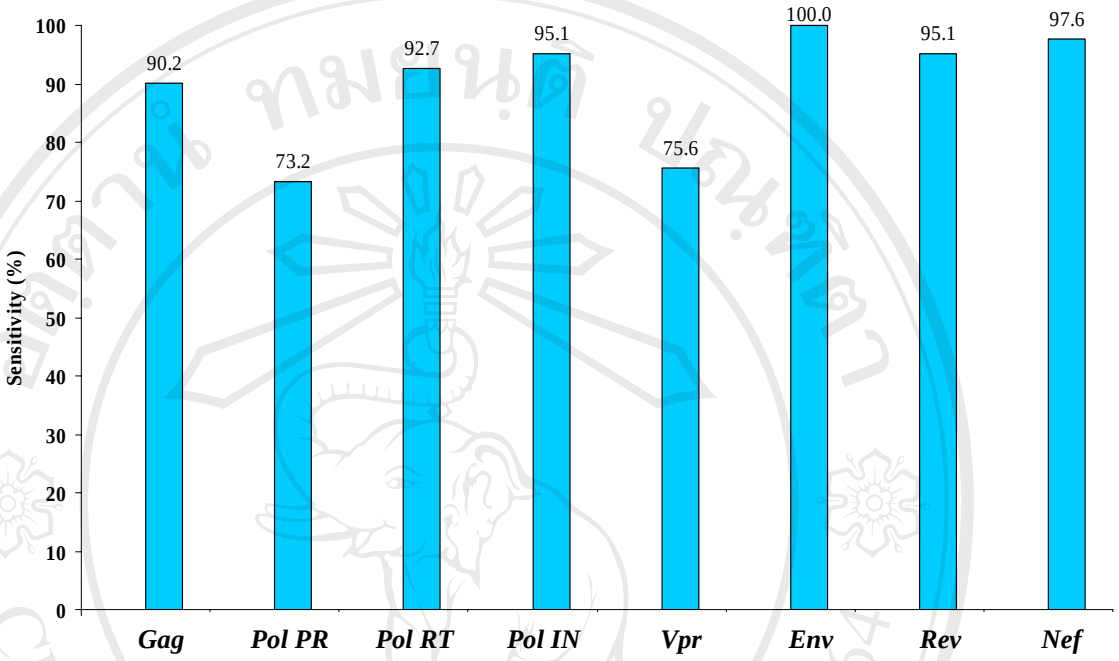

Figure 4.9 Sensitivity of the MSSP assay on the panel of 41 clinical DNA samples in each region. Each bar represents the number of expected results by region analyzed (n = 41). The sensitivity was highest in *Env* region 100%, and lowest in *Pol protease* 73%.

Table 4.3 Sensitivity and specificity of primers in MSSP assay

Region of evaluation	Total (n)	Number of expected results	Number of unexpected result	Number of PCR negative	Sensitivity[a] (%)	Specificity[b] (%)
Gag	41	37	0	4	90.2	100.0
Pol protease	41	30	0	11	73.2	100.0
Pol RT	41	38	0	3	92.7	100.0
Pol integrase	41	39	0	2	95.1	100.0
Vpr	41	31	0	10	75.6	100.0
Env	41	41	0	0	100.0	100.0
Rev	41	39	0	2	95.1	100.0
Nef	41	40	0	1	97.6	100.0
All regions	**328**	**295**	**0**	**33**	**89.9**	**100.0**

[a] Number of expected results divided by the total number of tests in each genome.

[b] No cross reactive were found.

4.4 Performance of Reverse Transcription MSSP assay on clinical samples

The assay was field tested on archived serum of 337 HIV-1 prevalence cases identified during 1999 to 2000 from the study "Epidemiology of HIV-1 among opiate users in northern Thailand: prevalence phase". The reactivity was in a range of 73-93% which were 79, 73, 89, 93, 90, 76, 90, and 91 % for *Gag (p17-p24), Pol (p2-p7 p6 protease),Pol (p51 RT), Pol (p31 integrase), Vpr, Env (gp120), Rev, Nef,* respectively, as shown in Figure 4.10 and Table 4.4. The results of Reverse Transcription MSSP assay of each sample in each genome region are shown in Appendix A. The numbers of typeable regions found were 0, 1, 2, 3, 4, 5, 6, 7 and 8. The percentage of the samples typed corresponding to the numbers of their typeable regions were 0.89, 0.59, 2.67, 2.37, 2.08, 7.72, 10.09, 29.38 and 44.21 %, respectively (Figure 4.11). With respect to the number of regions and the percentage of typeable samples, up to 99.1% were typed at least for 1 region and 44.2% had all eight regions typed. Interestingly, 93.5% of samples had at least 4 regions typed (Figure 4.12).

To practically authenticate a criterion for HIV-1 genotyping by the MSSP assay, subtype assignment was given to a sample when at least four regions were typeable. For samples with less than four typeable regions they were classified as non-typeable samples. These non-typeable strains were further classified as subtype-containing stains regarding to their typed regions or non-reactive samples. Under these criteria, the reactivity of MSSP assay was 93.5 %.

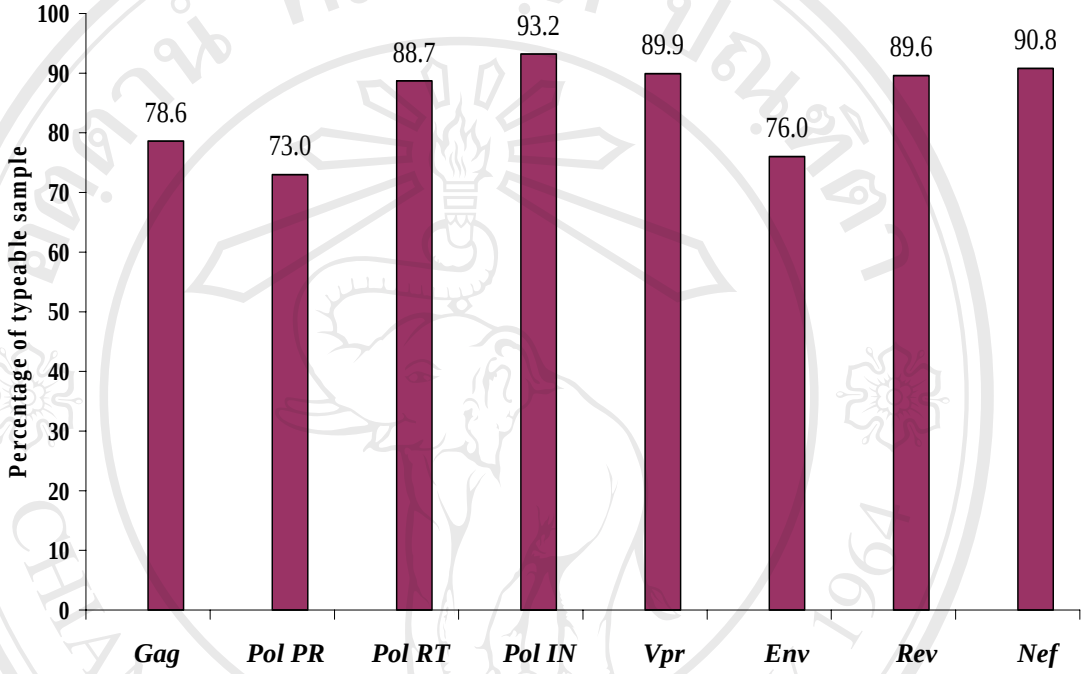

Figure 4.10 Reactivity of Reverse Transcription MSSP assay on 337 serum samples in each region. Each bar represents the total number of positive PCR reactions by region analyzed (n= 337). The reactivity was highest in *Pol integrase* region 93%, and lowest in *Pol protease* 73%.

Table 4.4 Reactivity of primers in drug users cohort using the Reverse Transcription MSSP assay

Region of Evaluation	Total (n)	PCR+	Reactivity[a]
Gag	337	265	78.6
Pol protease	337	246	73.0
Pol RT	337	299	88.7
Pol integrase	337	314	93.2
Vpr	337	303	89.9
Env	337	256	76.0
Rev	337	302	89.6
Nef	337	306	90.8
All regions	**2696**	**2291**	**85.0**

[a] Number of samples with positive PCR results divided by the total number of tests in each genome region.

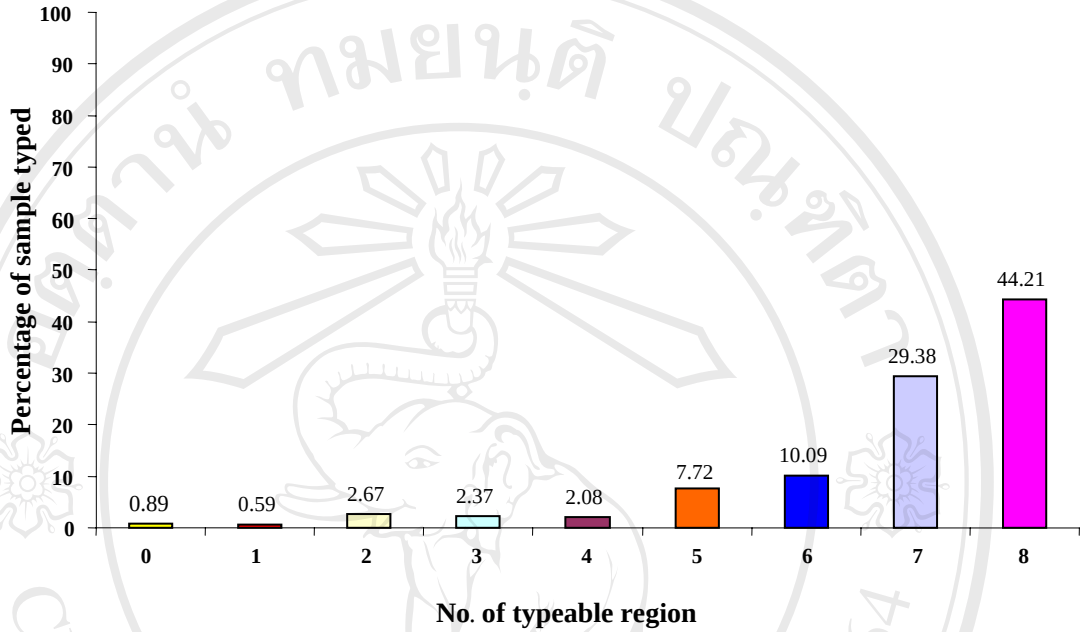

Figure 4.11 Percentage of sample typed by number of typeable region
Each bar represents the percentage of samples which were typed according to the number of typeable region.

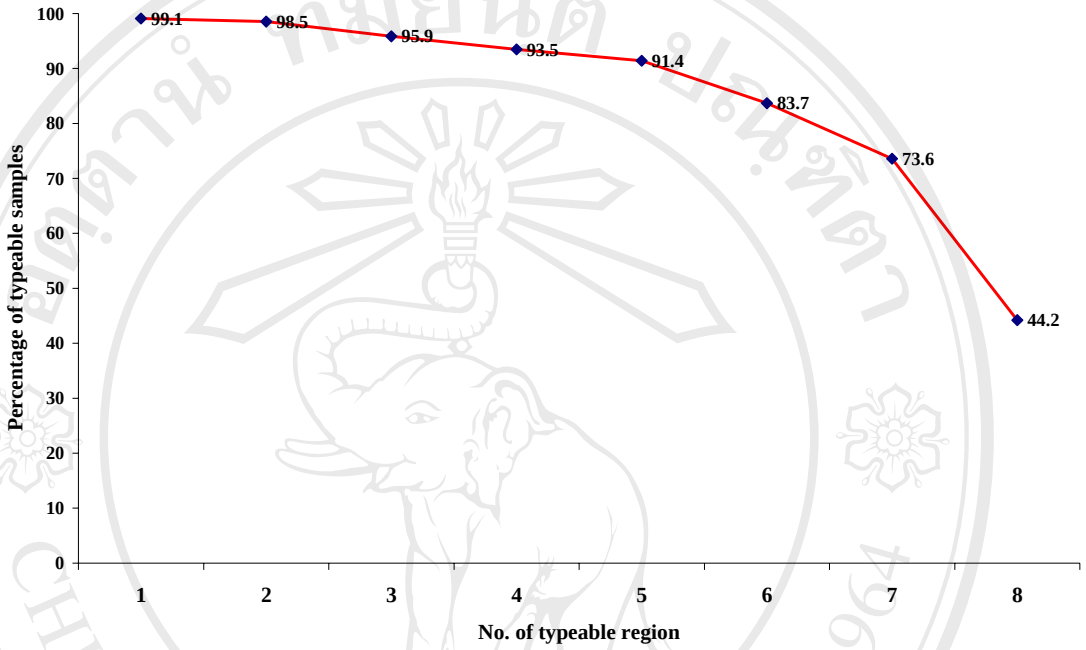

Figure 4.12 Percentage of typeable samples by number of typeable region. The line represents the percentage of samples which were typed according to the number of typeable regions.

In this cohort, there were 347 seropositive volunteers, but serum from 337 infected individuals was available for the assay. CRF01_AE was the most prevalent strain identified among the others. As shown in Figure 4.13, subtype distribution was 77.4% (n=261) CRF01_AE, 12.2% (n= 41) CRF01_AE /B recombinant, 3.3% (n=11) subtype B, and 0.6% (n=2) CRF01_AE/C recombinant and 7% (n=22) non-typeable samples. For non-typeable samples, they were 54.5% (n=12) CRF01_AE-containing strains, 22.7% (n=5) subtype B-containing strains, 4.6% (n=1) CRF01_AE /B -containing strain, 4.6% (n=1) subtype C -containing strain and 13.6% (n=3) non-reactive samples.

4.5 Reverse Transcription MSSP assay and dual infections

MSSP assay was also capable of detecting dual infections by PCR reactive with two subtypes in a given genome region. In this study, there were 14 out of 337 (4.2%) HIV-1 infected individuals identified as dual infections. There were 13 (3.8%) CRF01_AE/B and 1 (0.3%) CRF01_AE/C, and these 14 strains were among 43 recombinant strains identified in this cohort (Table 4.5). Among these 14 strains, OUR 1955 and OUR 2497 were further investigated by PCR, cloning and sequencing in Dr. McCutchan's laboratory at US Military HIV Research Program. OUR 1955 gave a dual reactive with both CRF01_AE and subtype B in *Pol RT* region and OUR 2497 gave a dual reactive with both CRF01_AE and subtype B in *Rev* region. Cloning and sequencing revealed that they both harbored both strains in the given region.

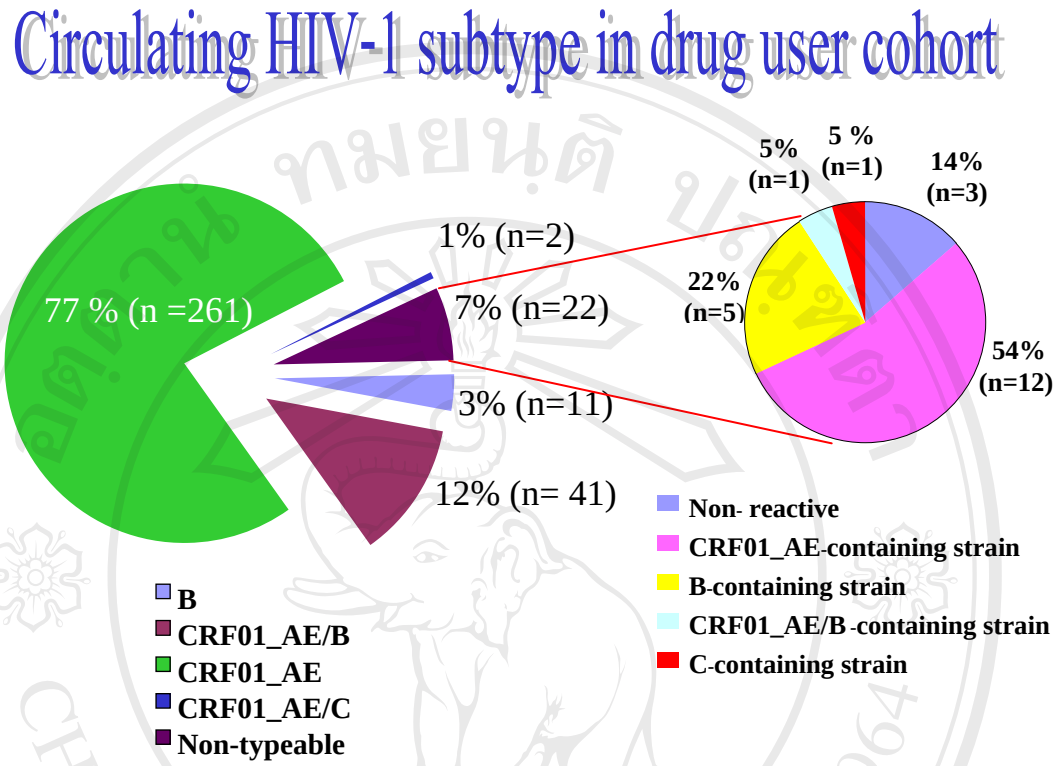

Figure 4.13 HIV-1 subtype distribution in drug user cohort. Among 337 available samples of HIV-1 infected volunteers in prevalence phase were tested by RT MSSP assay. Only samples that giving reactivity at least four regions were subtype designated or typeable. With this criterion, CRF01_AE was the most prevalent strains identified among the other. Subtype distribution was 77.4% (n=261) CRF01_AE, 12.2% (n= 41) CRF01_AE /B recombinant, 3.3% (n=11) subtype B, and 0.6% (n=2) CRF01_AE/C recombinant and 7% (n=22) non-typeable samples. For non-typeable samples, they were 54.5% (n=12) CRF01_AE-containing strains, 22.7% (n=5) subtype B-containing strains, 4.6% (n=1) CRF01_AE /B -containing strain, 4.6% (n=1) subtype C -containing strain and 13.6% (n=3) non-reactive samples.

Table 4.5 The results of Reverse Transcription MSSP assay in samples with dual infections

ID No.	No. of typeable region	Putative strain	Gag	Pol PR	Pol RT	Pol IN	Vpr	Env	Rev	Nef
Positive B	8	B	B	B	B	B	B	B	B	B
Positive C	8	C	C	C	C	C	C	C	C	C
Positive E	8	CRF01_AE	E	E	E	E	E	E	E	E
CE recombinant	8	CRF01_AE/C	C	C	C	C	E	E	E	E
BE recombinant	8	CRF01_AE/B	B	B	B	B	E	E	B	B
BE recombinant	8	CRF01_AE/B	E	B	B	E	E	E	E	E
Negative	0	Non-reactive								
800	5	CRF01_AE/B Dual		B	B		B		E	BE
403	6	CRF01_AE/B Dual		B	E	E		BE	E	BE
2266	6	CRF01_AE/B Dual		B	BE	E		E	E	E
1420	7	CRF01_AE/B Dual	E		E	E	E	BE	E	E
1955	7	CRF01_AE/B Dual		E	BE	E	E	E	E	E
354	8	CRF01_AE/B Dual	E	B	B	E	B	E	B	BE
357	8	CRF01_AE/B Dual	E	E	E	E	E	E	BE	BE
1913	8	CRF01_AE/B Dual	E	BE	E	E	E	E	E	E
1978	8	CRF01_AE/B Dual	E	E	BE	E	E	E	E	E
2114	8	CRF01_AE/B Dual	E	E	BE	E	E	E	B	E
2214	8	CRF01_AE/B Dual	E	B	BE	E	B	E	B	B
2497	8	CRF01_AE/B Dual	E	E	E	E	E	E	BE	E
2511	8	CRF01_AE/B Dual	E	BE	BE	E	BE	E	B	B
2429	6	CRF01_AE/C Dual	EC	E	E	E	E			E

This table shows the patterns of Reverse Transcription MSSP results of 14 samples with dual infection in eight regions including the patterns of recombinants, positive control, and negative control.

4.6 Verification of subtype B specific PCR products in *Pol RT* region

There were 5 samples (OUR 2114, OUR 2214, OUR 2266, OUR 2275 and OUR 2436) that subtype B specific PCR in *Pol RT* region gave a strong positive PCR product but with a larger molecular size than expected (Figure 4.14). The PCR products of OUR2114, 2214 and 2275 as show in Figure 4.14 were further investigated by cloning and sequencing in Dr. McCutchan's laboratory at US Military HIV Research Program. The sequences obtained as shown in Figure 4.15 were designated as HIV-1 subtype B by HIV-1 sequence locator and genotyping tool from Los Alamos HIV Sequence Database Website (http://www.hiv.lanl.gov). HXB2 numbers of these sequences were from 3042-3324. The sequences of genome region where the inner 3'-primer for subtype B sits were divert. The degree of mismatched was high enough to create primer-binding failure. The PCR products obtained must be the product from the inner 5'-primer and the outer 3'-primer that was left over from the first round and was confirmed by its molecular size (~280 bp).

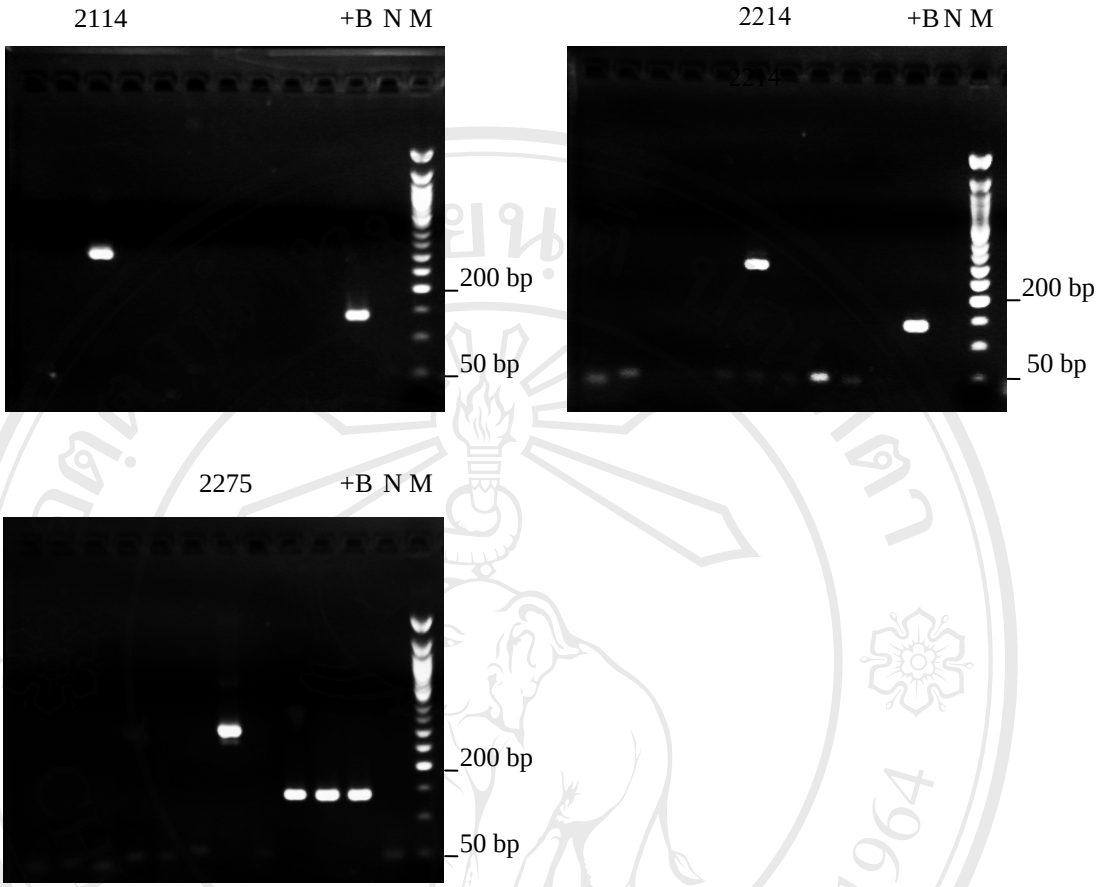

Figure 4.14 Gel picture of subtype B in *Pol RT* region. Detection of subtype B samples using subtype-specific primers for subtype B in *Pol RT* region. Product with the expected sized of 143 bp were seen in lane of subtype B positive control. A negative control (N) was included in the experiment. Lane M was 50 bp DNA markers. The positive products were about 280 bp.

Figure 4.15 DNA sequences of subtype B in *Pol RT* region. The *Pol RT* sequences of HIV-1 subtype B strains, OUR 2114, 2214 and 2275, aligned with the primers. The mismatches nucleotides between the primer and strains were found in the genome region where second round reverse primer located. The mismatches among the strains are in blue. The mismatches nucleotides between the primers and the strains are in red.

4.7 HIV-1 subtype distribution and socio/demographic characteristics of drug users in northern Thailand

HIV-1 subtyping and epidemiological data were summarized as shown in Table 4.6. Over all, among 337 HIV-1 infected individuals, 261 (77%) were CRF01_AE, 41 (12%) were CRF01_AE/B, 11 (3%) were subtype B, 2 (1%) were CRF01_AE/C, and 22 (7%) were non-typeable. CRF01_AE was predominant in age group between 20 and 29 years. It was also mostly prevalent among males, females, Thai citizens, hill tribes, the subjects with IDU history and the subjects with sexual exposures. CRF01_AE was associated with age between 20 and 29 years, while subtype B was not found in this age group. Interestingly, CRF01_AE/B recombinants were seen more common among age older than 40 years, especially in IDUs. The proportion of CRF01_AE/B was higher in female, whereas other strains were distributed evenly in both genders. Thai citizens had more subtype B than hill tribes, but CRF01_AE/C was identified in hill tribes only. None of subtype B was identified in non-IDU, while subtype B, CRF01_AE/B, and CRF01_AE/C were seen more in IDU. Non-typeable samples were found more among hill tribes. Ninety four percent of this population reported a history of sexual exposures. The history of sexual exposures was associated with subtype B infection but not with other HIV-1 strain infections. Statistical analysis of sociodemographic characteristics and HIV-1 subtype distribution will be further elaborated elsewhere (Kijak et al, in preparation).

Table 4.6 HIV-1 subtype distribution among drug users cohort in Northern Thailand, 1999-2000

Characteristic	Total samples		Subtype B		CRF01_AE		CRF01_AE/B		CRF01_AE/C		Non-typable	
	n	%	n	%	n	%	n	%	n	%	n	%
All samples	337	100	11	3	261	77	41	12	2	1	22	7
Age (years)												
<20	18	5	1	6	14	78	2	11	0	0	1	6
20-29	166	49	0	0	139	84	15	9	1	1	11	7
30-39	108	32	7	6	79	73	14	13	1	1	7	6
?40	45	13	3	7	29	64	10	22	0	0	3	7
Sex												
Female	23	7	1	4	15	65	5	22	0	0	2	9
Male	314	93	10	3	246	78	36	11	2	1	20	6
Ethnicity												
Thai	222	66	10	5	174	78	29	13	1	0	8	4
Hill tribe	115	34	1	1	87	76	12	10	1	1	14	12
Type of drug used												
Non-injecting	38	11	0	0	33	87	2	5	0	0	3	8
injecting	299	89	11	4	228	76	39	13	2	1	19	6
Having sex												
No	20	6	0	0	16	80	3	15	0	0	1	5
Yes	317	94	11	3	245	77	38	12	2	1	21	7